THE SOLUBILITIES OF SOME VERTEBRATE FIBRINOGENS IN PLASMA–ETHANOL MIXTURES[1]

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Fibrinogen, the plasma protein which forms fibrin under the action of thrombin, is the least soluble major component of plasma. It is a euglobulin precipitating when the ionic strength is reduced much below the physiological level and is "salted out" of mammalian plasmas at such relatively low ionic strengths as half saturated (3.2 *M*) sodium chloride (Hammarsten, 1879; Florkin, 1930) and 10.6 per cent (0.75 *M*) sodium sulfate (Howe, 1923). It is also precipitated in dilute solutions of ethanol at low temperatures (Edsall, Ferry and Armstrong, 1944; Morrison, Edsall and Miller, 1948) and we have used this behavior as a criterion of solubility.

Materials and Methods

Fibrinogens from representatives of four vertebrate classes were studied. Blood was usually obtained by exposure of the heart and direct heart puncture, although some animals were bled by heart puncture through the chest and others from the canulated carotid artery. Siliconed needles and syringes containing a tenth volume of 10 per cent sodium citrate as an anticoagulant were used. Blood was chilled on ice usually within 10 minutes and stored in siliconed or plastic containers until centrifugation, which was carried out in two stages with the second at 5000 r.p.m. to insure removal of fine particulate material which would otherwise be carried down in the clot. This procedure usually produced a very clear straw or yellow plasma although in certain individual frogs and turtles a substantial amount of hemoglobin was present in the plasma even with these precautions. This hemolysis was not correlated with differences in technique, so it may represent differences in the physiological condition of the animals about whose background little was known.

Since as little as one cc. of blood was the most that could be obtained from certain of the smaller animals, it was necessary to adapt procedures to small volumes. One cc. of plasma was diluted with an equal volume at an appropriate concentration of ethanol (by volume) and sodium chloride to maintain the ionic strength constant at 0.15 and the mixture was equilibrated for two hours at 0° C. The precipitate and supernatant phases were then separated by refrigerated centrifugation in the same tube and decantation. The precipitate was dissolved in 2½ cc. of pH 6.4 phosphate buffer, ionic strength of 0.15, and two 1 cc. samples of this solution together with a 1 cc. sample from the supernatant were analysed. The fibrinogen was assayed gravimetrically after clotting with thrombin, syneresis and washing, drying and weighing

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(Morrison, 1947). Since the final clot weights usually ranged from 0.1 to 1 mg., a torsion balance sensitive to 0.005 mg. was employed. With these small amounts results usually agreed within 5 per cent. This represents a substantially greater error than had been obtained with 20 mg. clots (Morrison, 1947) of human fibrinogen. For mammalian fibrinogens a final concentration of 0.1 to 0.2 unit (Ferry and Morrison, 1947) per cc. of bovine thrombin [2] was employed. Bovine thrombin was also employed to clot fibrinogens of lower forms but since the heterologous reaction between fibrinogens and thrombins from different classes is much less effective (unpublished observations) larger amounts were necessary. A concentration sufficient to yield a 5 to 10 minute clotting time was usually employed and clotting was continued for two to four hours. Considerable fibrinolytic activity was sometimes encountered in plasmas from the lower forms and it was necessary to discard such series.

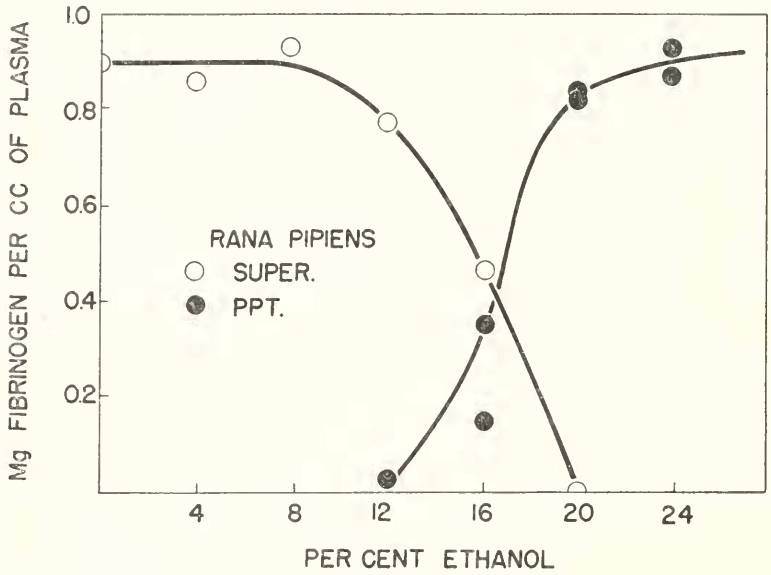

FIGURE 1. A representative series on 1 cc. samples of plasma showing the distribution of fibrinogen in the precipitate and supernatant phases with increasing amounts of ethanol. Open circles, supernatant phase; closed circles, precipitate phase.

Figure 1, showing the yield of fibrinogen in the supernatant and precipitate as a function of ethanol concentration, illustrates a typical experimental series on frog plasma using a total of 7 cc. of plasma. The sum of the supernatant and precipitate values usually was satisfactorily constant although at low fibrinogen levels low yields will be obtained (Morrison, 1947). For example in Figure 1 at 12 per cent ethanol this sum is only 90 per cent of the expected value. At high ethanol concentrations the yields tended to rise above the initial value presumably because of precipitation and/or denaturation of other plasma components with subsequent occlusion in the clot. In such cases the synerized clots were distinctly yellower.

[2] This material was supplied through the kindness of Dr. James Lesh of Armour and Company.

Results and Discussion

Precipitation curves for three species of mammals, the rabbit, dog and cow, are shown in Figure 2. The points are sufficiently homogeneous for a single curve to represent the three species. Actually, successive series on individuals yielded as much variation as is shown between species in this figure. Human fibrinogen, which is 65 per cent precipitated at 8 per cent ethanol, fits quite well with these series.

Results on two turtles, the snapping turtle, *Chelydra serpentia* and the terrapin, *Pseudemys,* are shown in Figure 3. Reasonable correspondence is seen between individuals but there is a distinct difference between the two genera.

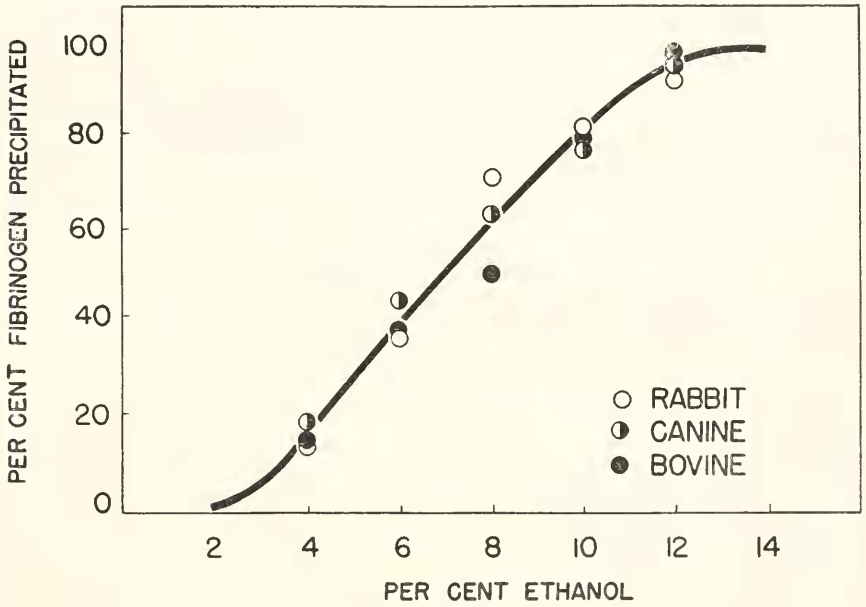

Figure 2. The precipitation of mammalian fibrinogens from their plasmas (per cent of the plasma level) as a function of ethanol concentration at 0° C. Open circles, rabbit; half circles, canine; closed circles, bovine. Each of the values for rabbit and dog represents an average of two series on different individuals.

Results on three amphibia, the bullfrog, *Rana catesbiana,* the green frog, *Rana pipiens,* and the mudpuppy, *Necturus,* are shown in Figure 4. Again, individuals of the same species fall together but distinct differences are seen between groups. The curve for the bullfrog suggests that two components with different properties were being precipitated, since 5 to 15 per cent of the material came down between 4 and 8 per cent ethanol while the bulk of the fibrinogen required considerably higher amounts of alcohol for precipitation.

Average curves for the species studied are assembled in Figure 5. The shapes of these curves are very similar although minor differences may be noted. It is immediately seen that the precipitation curves for the several classes stand in the same regular series which relates them phylogenetically, the mammalian fibrinogen

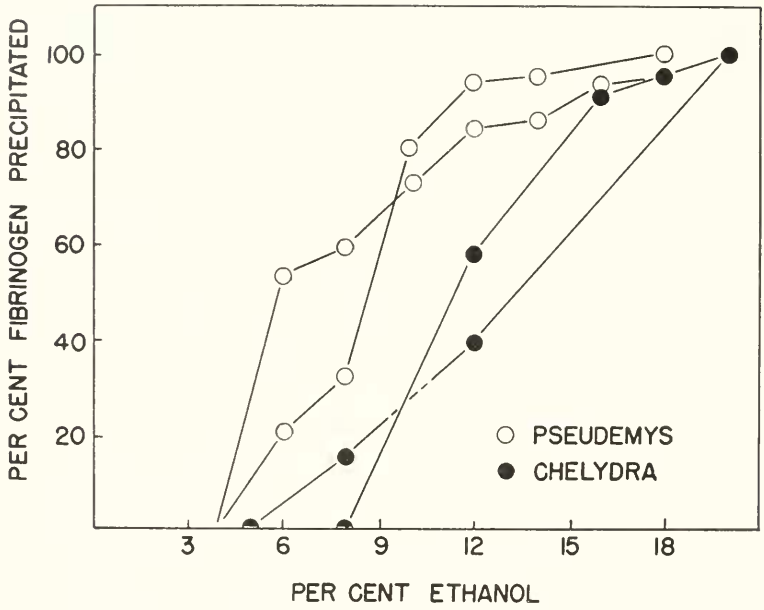

FIGURE 3. The precipitation of fibrinogen from two species of turtle plasma as a function of ethanol concentration at 0° C. Closed circles, snapping turtle, *Chelydra serpentina;* open circles, terrapin, *Pseudemys.*

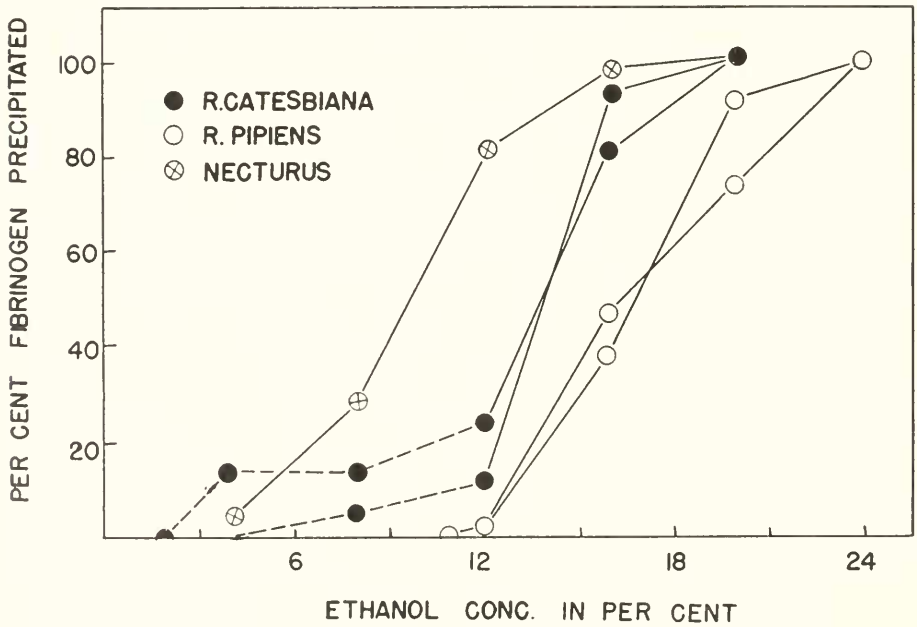

FIGURE 4. Precipitation of several amphibian fibrinogens from plasma as a function of ethanol concentration at 0° C. Open circles, grass frog, *Rana pipiens;* closed circles, bullfrog, *Rana catesbiana;* cross circles, mudpuppy, *Necturus.*

TABLE I

Average ethanol concentrations for precipitation of various fibrinogens from plasma

Group	Species	Series	% Ethanol for	
			50% ppt.	80% ppt.
Mammals	3	5	7	9
Turtles	2	4	11	13
Frogs	2	4	15	18
Carp	1	1	19	24

being least, and the fish fibrinogen (carp) most soluble. The ethanol concentrations required for 50 and 80 per cent precipitation, respectively, are summarized in Table I. Although the broad trend is evident, differences between species are of the same order of magnitude as are the differences in average values of the classes. And, indeed, the curve for the amphibian, *Necturus*, which is not indicated on Figure 5, falls more nearly with the turtles than with the frogs.

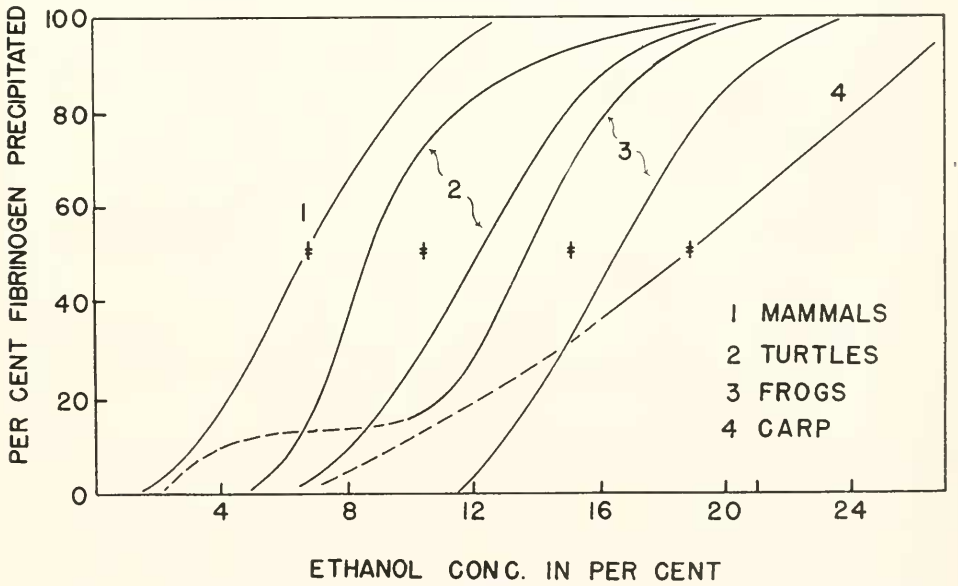

FIGURE 5. Average curves for the precipitation of fibrinogen as a function of ethanol concentration for the species shown in Figures 2, 3, and 4, together with a single curve for the carp. Symbols show the average values for 50 per cent precipitation for the four classes.

Although these precipitation curves reflect changes in solubility, they are not the same as solubility measurements since they will be influenced by the amount of the fibrinogen originally present in the plasma. However, since the curves are quite steep, variation in initial fibrinogen level will have a relatively small effect on the ethanol concentration required for 50 per cent precipitation. In Figure 6 the average results of measurements on the three classes have been presented in terms

of actual solubility of fibrinogen in the supernatant phase at successive ethanol concentrations. The trend toward increasing solubility in the lower forms is again shown.

It is worth noting that this trend in fibrinogen solubility appeared to be followed by the other components since the mass of the fibrinogen-containing precipitate in the lower forms was not substantially larger than in the higher forms despite the considerably higher ethanol concentration. Plasma samples from two primitive fish, the garpike and the bowfin, which were unsatisfactory for fibrinogen assay, showed almost no precipitation even at 25 per cent ethanol.

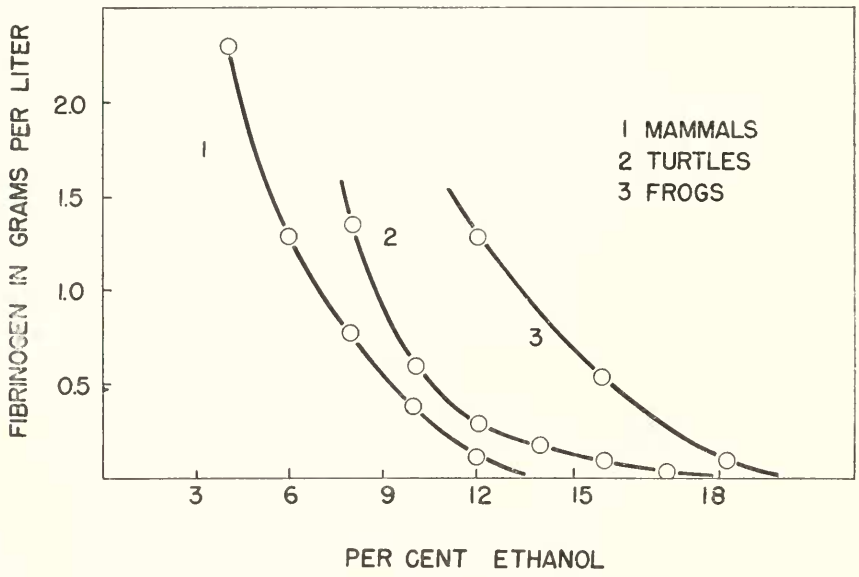

FIGURE 6. Average solubility curves for (1) mammal, (2) turtle, and (3) frog fibrinogens calculated from the supernatant values of the series in Figures 2, 3 and 4.

The significance of these differences in solubility in terms of molecular structure cannot be directly predicted. A more soluble molecule may also be a smaller molecule if the two are similar in other respects. Thus, in heterogeneous mixtures of high polymers the lower molecular weight fractions are the more soluble and, similarly, the products of protein cleavage are more soluble than the original protein. Besides their functional similarity, electrophoretic evidence suggests that the fibrinogens may be chemically similar. Deutsch and Goodloe (1945) reported a mobility of 2.6×10^{-5} cm.^{2} v^{-1} sec.$^{-1}$ for carp fibrinogen as compared to an average value of 2.43 (range: 2.1 to 3.2) for 12 species of mammals. Deutsch and McShan (1949) found no general shift toward components of higher mobility in studies on serum proteins from snakes, frogs and fish. However, their carp serum showed a shift toward components of higher mobility, perhaps correlated with the high albumin to globulin ratio reported for this species by Field, Elvehjem and Juday (1943). Such behavior, by reflecting a higher net charge on the molecule, might also account for the observed differences in solubility. Another piece of evidence which may suggest

smaller molecules in the lower forms is the fact that the clots formed from these fibrinogens are invariably of the "fine" variety (Ferry and Morrison, 1947) showing little turbidity and synerizing with difficulty. These properties are considered to indicate small units of structure and small interstices such as might be expected from an aggregation of smaller molecules. Final conclusions must, however, await more careful characterization of these fibrinogen molecules by physical and other means.

Summary

The precipitation and solubility of fibrinogens from plasmas of four vertebrate classes by ethanol at 0° C. and 0.15 ionic strength was investigated. A regular series showing increasing solubility was observed as one went from mammals down through the lower classes.

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